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Outbreak of Intestinal Infection Due to *Rhizopus microsporus*[▽]

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Sinopulmonary and rhinocerebral zygomycosis has been increasingly found in patients with hematological malignancies and bone marrow transplantation, but intestinal zygomycosis remains very rare in the literature. We investigated an outbreak of intestinal infection due to *Rhizopus microsporus* in 12 patients on treatment for hematological malignancies over a period of 6 months in a teaching hospital. The intake of allopurinol during hospitalization ($P < 0.001$) and that of commercially packaged ready-to-eat food items in the preceding 2 weeks ($P < 0.001$) were found to be independently significant risk factors for the development of intestinal zygomycosis. A total of 709 specimens, including 378 environmental and air samples, 181 food samples, and 150 drug samples, were taken for fungal culture. Among them, 16 samples of allopurinol tablets, 3 prepackaged ready-to-eat food items, and 1 pair of wooden chopsticks were positive for *Rhizopus microsporus*, which was confirmed by ITS1-5.8S-ITS2 rRNA gene cluster (internal transcribed spacer [ITS]) sequencing. The mean viable fungal counts of allopurinol obtained from wards and pharmacy were 4.22×10^3 CFU/g of tablet (range, 3.07×10^3 to 5.48×10^3) and 3.24×10^3 CFU/g of tablet (range, 2.68×10^3 to 3.72×10^3), respectively, which were much higher than the mean count of 2×10^2 CFU/g of food. Phylogenetic analysis by ITS sequencing showed multiple clones from isolates of contaminated allopurinol tablets and ready-to-eat food, of which some were identical to patients' isolates, and with one isolate in the cornstarch used as an excipient for manufacture of this drug. We attempted to type the isolates by random amplification of polymorphic DNA analysis, with limited evidence of clonal distribution. The primary source of the contaminating fungus was likely to be the cornstarch used in the manufacturing of allopurinol tablets or ready-to-eat food. *Rhizopus microsporus* is thermotolerant and can multiply even at 50°C. The long holding time of the intermediates during the manufacturing process of allopurinol amplified the fungal load. Microbiological monitoring of drugs manufactured for highly immunosuppressed patients should be considered.

Zygomycosis has become an important emerging infection in patients with hematological malignancy undergoing chemotherapy or bone marrow transplantation (24), especially with the availability of voriconazole as antifungal prophylaxis (25, 28, 44). Breakthrough zygomycosis in patients on voriconazole prophylactic treatment is usually manifested as sinopulmonary or rhinocerebral disease, since the fungal spores are ubiquitously found in the environment and could therefore be acquired through inhalation or traumatic inoculation through the skin or mucosa. Gastrointestinal zygomycosis has been a rare clinical entity (50, 54), but cases or outbreaks of tongue, gastric, or cutaneous zygomycosis due to *Rhizopus microsporus* after exposure to contaminated wooden tongue depressors have been reported (23, 26, 33).

The *Rhizopus microsporus* species group, which belongs to the class *Zygomycetes* and the order *Mucorales*, can cause life-threatening infections in immunocompromised patients (9, 39, 46). The laboratory diagnosis of *R. microsporus* relies mainly on the characteristic microscopic and phenotypic features of the fungal mycelium culture on agar medium. A molecular diagnostic test for this fungus is not widely available. PCR amplification and sequencing of the 18S and 28S rRNA genes (58, 64), internal transcribed spacer (ITS) region of rRNA (27), and recently PCR restriction fragment length polymorphism (22) and high-affinity iron permease gene sequence have been used to identify members of the clinically important *Zygomycetes* (30).

Here we report an unprecedented outbreak of intestinal zygomycosis due to *R. microsporus* as a result of the oral intake of contaminated allopurinol tablets and ready-to-eat food items in patients with hematological malignancies undergoing chemotherapy and bone marrow transplantation. The clinical spectrum of this outbreak of intestinal infection by *R. microsporus* ranges from asymptomatic colonization to mucosal involvement to invasive disease.

MATERIALS AND METHODS

Setting. The hematological oncology unit at Queen Mary Hospital, a university-affiliated teaching hospital in Hong Kong, serves as a tertiary referral center for treatment of refractory hematological malignancies and bone marrow transplantation. During the outbreak period, there were two pediatric (wards C6 and K8N) and two adult (wards K20N and J8N) medical wards providing care for these patients or for bone marrow transplantation. Except for C6, all these wards were equipped with positive-pressure high-efficiency particulate-filtered air in

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protective isolation rooms (K8N with 7 rooms, K20N with 14 rooms, and J8N with 10 rooms) for patients with neutropenia.

Outbreak investigation. An outbreak of intestinal infection due to *R. microsporus* occurred among these inpatients over a period of 6 months. Invasive intestinal zygomycosis was defined as a symptomatic case having an intestinal tissue specimen showing characteristic histological tissue invasion by aseptate hyphae with wide but varying diameter and a positive culture for *R. microsporus* with or without extraintestinal dissemination. Mucosal zygomycosis was defined as a case with abdominal symptoms, stool culture positive for *R. microsporus*, and radiological evidence of bowel mucosal thickening but without histological proof of tissue invasion. A case of colonization was defined as an asymptomatic case with surveillance stool culture positive for *R. microsporus*. Microbiological laboratory data were also retrieved to identify any unrecognized case and the past incidence of this rare infection. The medical records of the case patients were reviewed by clinical microbiologists and the infection control team as described in our previous outbreak investigations (5, 6, 7, 66). The hematologists were interviewed regarding any change in the chemotherapeutic regimen which might have altered the degree of immunosuppression in this group of patients, and the nursing staffs were inspected for any changes in patient care practice. Patients and their relatives, if available, were interviewed in detail regarding food, drinks, eating utensils, and drug history. A case control study was performed to identify the risk factors for the outbreak. Since their immunocompromised state is a known risk factor for zygomycosis, patients staying in the hematology and bone marrow transplant units with stool culture negative for the fungus were chosen as the control.

Screening of stool samples. During the period of the outbreak investigation, stool samples from all hospitalized patients being tested for bacterial culture in Queen Mary Hospital were also screened for *Mucorales*. To avoid potential contamination by wooden spatulas used during specimen collections (15, 57), stool samples were collected with sterile swabs and transported in sterile bottles. Pea-sized, formed stool of about 1 g or 0.5 ml of watery stool was streaked onto Sabouraud dextrose agar plus chloramphenicol (50 mg/liter) (Becton Dickinson and Company) and incubated at 37°C for 7 days.

Screening of environmental, food, and drug samples. Environmental samples from clinical areas, the kitchen, and the pharmacy and air samples from clinical areas, the kitchen, and the corridors were collected for fungal culture. Food items including fresh fruits, prepackaged ready-to-eat food, and taste additives were tested as well. Food items were cultured as described previously with modifications (51, 52). Tested items and swabs were inoculated into enrichment broth (containing peptone [10 g/liter], yeast extract [1 g/liter], dextrose [25 g/liter], and chloramphenicol [50 mg/liter]) and Sabouraud agar for incubation at 37°C. All broths were checked for mycelium formation every 24 h and subcultured on Sabouraud dextrose agar at day 5 for 48 h of incubation at 37°C. Quantitative culture was performed for the unopened refrigerated items of the same batch number if there were positives on culture for *Rhizopus* spp.

For screening of drugs, after swabs for fungal culture were taken from the internal surface of an opened vial, three tablets from each bottle were immersed in enrichment broth (containing peptone [10 g/liter], yeast extract [1 g/liter], dextrose [25 g/liter], and chloramphenicol [50 mg/liter]) incubated at 37°C for up to 7 days. Aliquots were taken for subculture on Sabouraud agar either if the broth became turbid or after 7 days, whichever was earlier. Quantitation of mold was performed by dissolving one tablet in 1 ml of enrichment broth, dividing into 10-μl aliquots in triplicate, and spreading on Sabouraud dextrose agar with modifications as described previously (53). The mean CFU count was the average of those from three different tablets in the same batch. The result was expressed as CFU/g of tablet.

Laboratory identification of *Rhizopus* isolates. Fungal colonies were examined under direct microscopy for morphological characteristic of *Rhizopus* spp. Isolates of the *R. microsporus* group were identified according to standard morphological criteria (17) and stored in 1 ml of sterile water at room temperature for genotypic analysis. Scanning electron microscopy was performed to identify the variety of *R. microsporus* group isolates.

Antifungal susceptibility and treatment outcome. Susceptibilities of *R. microsporus* to amphotericin B and posaconazole were determined by Etest (AB Biodisk, Sweden) according to the manufacturer's instructions. *Aspergillus flavus* ATCC 204304 was used as a control. The treatment outcome of our cases was recorded.

DNA extraction. Fungal DNA extraction was performed as described in our previous publications (60, 61). Briefly, DNA was extracted from 1 g of fungal cells in 10 ml of distilled water using the DNeasy plant minikit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The extracted DNA was eluted in 50 μl of buffer AE (10 mM Tris-Cl, 0.5 mM EDTA [pH 9.0]); the resultant mixture was diluted 10 times, and 1 μl of the diluted extract was used for PCR.

ITS1-5.8S-ITS2 rRNA gene cluster (ITS) sequencing. PCR amplification and DNA sequencing of the ITS were performed according to published protocols (59, 62). Briefly, DNase I-treated distilled water and PCR master mix, which contains deoxynucleoside triphosphates (dNTPs), PCR buffer, and *Taq* polymerase, were used in all PCRs by adding 1 U of DNase I (Roche) to 40 μl of distilled water or PCR master mix. The mixture was incubated at 25°C for 15 min and subsequently at 95°C for 10 min to inactivate the DNase I. The fungal DNA extract and controls were amplified with 0.5 μM primers (ITS1, 5′-TCCGTAG GTGAACCTGCGG-3′; and ITS4, 5′-TCCTCCGCTTATTGATATGC-3′) (Gibco BRL, Rockville, MD). The PCR mixture (25 μl) contained fungal DNA, PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2 mM $MgCl_2$, and 0.01% gelatin), 200 μM [each] dNTP, and 1.0 U *Taq* polymerase (Applied Biosystems, Foster City, CA). The mixtures were amplified using 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 10 min in an automated thermal cycler (Applied Biosystems, Foster City, CA). DNase I-treated distilled water was used as the negative control. Ten microliters of each amplified product was electrophoresed in 1.5% (wt/vol) agarose gel, with a molecular size marker (φX174 HaeIII digest; Boehringer Mannheim, Germany) in parallel. Electrophoresis in Tris-borate-EDTA buffer was performed at 100 V for 1.5 h. The gel was stained with ethidium bromide (0.5 μg/ml) for 15 min, rinsed, and photographed under UV light illumination.

The PCR products were purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Both strands of the PCR products were sequenced with an ABI Prism 3700 DNA analyzer (Applied Biosystems), using the PCR primers. The sequences of the PCR products were compared with sequences of closely related species in GenBank by multiple sequence alignment using the Clustal_X 1.83 software program (49).

Phylogenetic characterization. Phylogenetic tree construction was performed using the neighbor-joining method with Clustal_X 1.83. A 648-bp segment of ITS was included in the analysis.

Typing by random amplification of polymorphic DNA (RAPD). The DNA of the *Rhizopus* isolates was extracted using the PrepMan Ultra sample preparation kit (Applied Biosystems) according to the manufacturer's instructions. PCR was carried out as described previously with modifications (55). Reaction mixtures contained 1× PCR buffer, 3 mM $MgCl_2$, 200 μM (each) dNTP, 0.2 μM primer, 0.625 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 50 ng of genomic DNA in a 25-μl volume. Amplification was carried out in a GeneAmp 9700 thermal cycler (Applied Biosystems) with cycle conditions as follows: 95°C hot start for 10 min and 45 cycles of 92°C for 1 min, 35°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 7 min. Ten μl of PCR products from each reaction were analyzed by electrophoresis in 1.2% agarose gel in 1× Tris-borate-EDTA buffer and stained with 1× SYBR gold nucleic acid stain (Invitrogen).

RAPD banding patterns were compared based on the presence or absence of observed amplification products in gel electrophoresis. Numerical analysis was performed using the GelCompar II software program, version 3.0 (Applied Maths). Cluster analysis was performed with calculation of Jaccard coefficients, and a dendrogram was constructed using the unweighted pair-group method using arithmetic averages. However, no interpretative standards for typing of filamentous fungus by RAPD currently exist.

Statistical analysis. Statistical analysis was carried out to show the absence of differences in the clinical characteristics between the patient and control groups, apart from the risk factors proposed. The nonparametric Mann-Whitney U test was chosen for the continuous variables because there is no assumption of normal distribution of values in the sample variable as distinct from parametric tests. Fisher's exact test was used to compare independent categorical variables between groups and is considered superior to the chi-square goodness-of-fit test because it does not utilize a normal approximation to the binomial distribution, which could lead to errors when the sample numbers are small. All reported *P* values were two sided. A *P* value of <0.05 was considered statistically significant. Computation was performed using the SPSS (Statistical Package for the Social Sciences) software program, version 15.0 for Windows.

RESULTS

Outbreak investigation. Since the opening of a bone marrow transplant center at Queen Mary Hospital in 1990, a total of 44,940 fungal surveillance cultures, including 5,319 stool samples, were performed. Up to 31 December 2005, only 1 *Mucorales* isolate (an *Absidia* sp.) was recovered, from a sputum

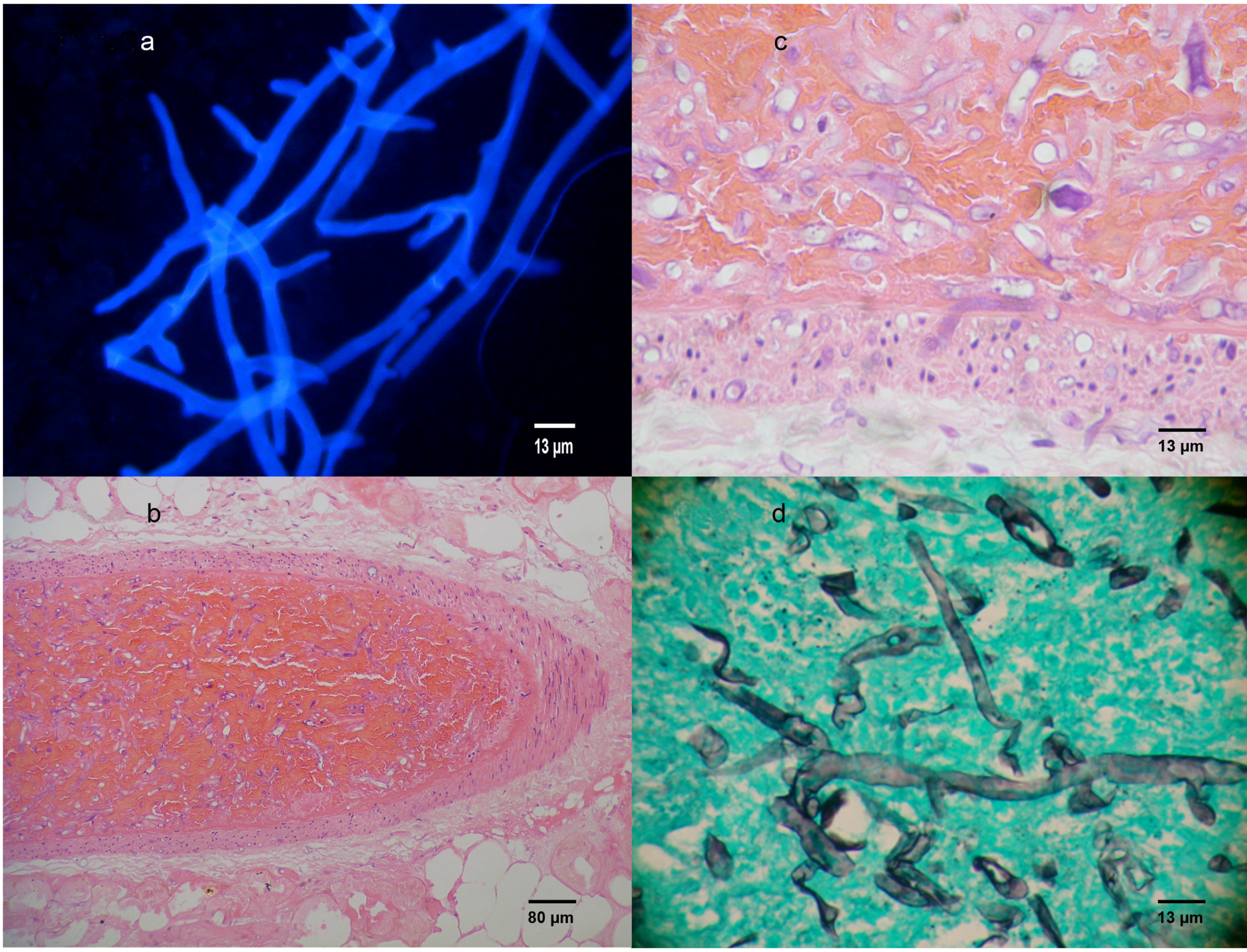

FIG. 1. Microbiological and histopathological examination of the resected intestinal specimen of case 5. (a) Potassium hydroxide (KOH)-digested wet mount of tissue stained by calcofluor white, showing bluish-white fluorescence of branching fungal elements without septum. (b) Hematoxylin-and-eosin-stained section of the mesoappendix with a vessel showing extensive thrombosis, with the lumen filled by blood cells and fungal elements. (c) Pale-staining hyphae appear as holes or bubbles in the section. Only red cells are seen in the thrombosed vessel due to severe neutropenia. Invasion into the vessel wall by fungal hyphae is seen. (d) Grocott silver-stained section of a vessel, showing wide wavy ribbon-like aseptate hyphae with broad-angle branching.

sample in the year 2004. During the same period, a total of 100,312 fungal cultures were performed for non-bone marrow transplant patients. Twenty-five specimens from 11 patients were positive for *Mucorales*, but none of them was isolated from stool or intestinal tissue. During the outbreak period, 14 surveillance stool samples from 7 patients out of a total of 557 stool samples were found to have *R. microsporus.* For septic workup of symptomatic cases, 1 wound swab from 1 patient, 1 abdominal fluid sample from 1 patient, and 20 operative tissue specimens from 3 patients were obtained. After the retrospective review of clinical and laboratory data, a total of 12 cases (5 invasive, 2 mucosal, and 5 colonization) due to *R. microsporus* were identified during the study period (Fig. 1a to d). They were of ages between 6 and 73 years, and the male-to-female ratio was 9:3 (Table 1). All patients had solely intestinal involvement without clinical or radiological evidence of sinopulmonary involvement. The cases were scattered in the four hematology-oncology wards (C6, K8N, K20N, and J8N) and in one other general adult medical ward (E3), where these patients may stay temporarily before moving into hematology-oncology wards. In a case control study, the use of allopurinol during hospitalization and the intake of commercially packaged ready-to-eat food in the preceding 2 weeks were found to be independent statistically significant risk factors for developing intestinal infection by *R. microsporus* (Table 2).

A total of 709 nonpatient specimens were obtained for fungal culture. These included 378 environmental and air samples taken from the wards (246 samples), pharmacy (17 samples), kitchen and catering service (63 samples), storeroom (40 samples), corridor (5 samples), and microbiology laboratory (7 samples). Another 181 food samples and 150 drug samples were also tested (Table 3). Cultures revealed *Paecilomyces* species (8 samples), *Penicillium* species (8 samples), *Exophiala* species (3 samples), *Aspergillus niger* (5 samples), *Aspergillus fumigatus* (1 sample), *Absidia* species (1 sample), and *Rhizopus microsporus* (1 sample) from environmental specimens, 5 sam-

TABLE 1. Clinical characteristics of patients with intestinal zygomycosis due to *Rhizopus microsporus* isolated in operative tissue or stool samples

Case (type)[a]	Sex/age (ward)[b]	Hemic malignancy (chemotherapy)[c]	Symptoms and key blood tests[d]	Abdominal CT and laparotomy findings[e]	Histopathological findings[f]	Antifungal regimen and clinical outcome[g]
1 (I)	M/6 (C6)	ALL (V, D, Met, Leu, and Dexa)	Abdominal pain; ANC: 0.34; ALT: 13; Cr: 44	CT: distended bowel loops at central abdominal region, swollen R psoas muscle with abscess formation; moderate R hydronephrosis; laparotomy: necrotic small, large bowel, R posas and paraspinal muscles	Extensive involvement of all the tissues by fungal elements with pronounced angiotrophism with thrombosis, invasion of the blood vessel wall by fungal hyphae, and extensive full-thickness infarction of the bowel walls	Posa, AmB, Caspo 3 days and Des 27 days after symptom onset; died 36 days after symptom onset
2 (I)	M/11 (K8N)	AML (Met, Ida, Cyt and E)	RLQ pain; ANC: 0.05; ALT: 9; Cr: 48	CT: swollen appendix measuring up to 2.5 cm with thickened wall of 7 mm; laparotomy: infarction and perforation of paracecal appendix and adjacent omentum	Transmural infarction of the appendix with an abundant amt of fungal organisms suggestive of *Mucorales* by their branching at right angles and invasion of blood vessel walls	Posa, AmB, Caspo, Des 1 day after symptom onset; fever and abdominal pain resolved 16 days after therapy
3 (I)!	M/57 (E3)	DLBCL (V, D, Met, A, and Dexa)	Abdominal pain; ANC: 0.26; ALT: 152; Cr: 126	CT: intraperitoneal gas suggestive of perforated bowel; laparotomy: two perforations at cecum with indurated edge	Mucosal infiltration and necrosis due to fungal element	Fluconazole; died 8 days after symptom onset
4 (M)	M/35 (J8N)	Fanconi's anemia/MDS (TBI, ATG, F, and Cyc)	Diarrhea; ANC: 0.48; ALT: 57; Cr: 67	CT: segmental thickening of proximal jejunal wall	NA	Posa, AmB, anidulafungin 1 day after symptom onset; died 44 days after symptom onset due to complications of BMT
5 (I)	M/38 (K20N)	ALL (Clo)	RLQ pain; ANC: 0.03; ALT: 833; Cr: 94	CT: bowel wall thickening of the terminal ileum, cecum, and proximal ascending colon; laparotomy: swollen cecum and terminal ileum; acute gangrenous appendicitis	Extensive invasion of the terminal ileum, cecum, appendix, and omentum by fungal elements composed of aseptate hyphae; fungi stain with Grocott silver stain and Periodic acid-Schiff-digested stain; morphologically consistent with *Mucorales*	Posa, AmB, Caspo 3 days after symptom onset; died 28 days after symptom onset
6 (C)	M/38 (J8N)	AML (B and Cyc)	Asymptomatic; ANC: 6.54; ALT: 37; Cr: 68	CT: no abnormal bowel dilatation or bowel wall thickening	NA	Posa 5 days after isolation of *Rhizopus sp.* in stool; stable
7 (C)	M/50 (J8N)	Precursor T-ALL (TBI + Cyc)	Asymptomatic; ANC: 2.73; ALT: 60 Cr: 67	CT: no abnormal bowel dilatation or bowel wall thickening	NA	Posa 2 days after isolation of *Rhizopus sp.* in stool; stable
8 (I)!	F/42 (K20N)	NK cell lymphoma (A)	Abdominal distention; ANC: 3.72; ALT 12; Cr 79	CT: moderate amt of ascites, thickened peritoneal surface with omental cake suggestive of lymphomatous infiltration; laparotomy: thinning of cecal wall without perforation; inflamed omentum with adhesion to pelvic cavity	Extensive involvement of small intestine, stomach, urinary bladder, omentum, mesentery, large intestine, kidney, lung, liver, spleen, and pancreas with branching aseptate fungal elements	Voriconazole; died 23 days after symptom onset; *Rhizopus sp.* isolated in peritoneal fluid 1 day after patient succumbed
9 (C)	M/73 (K20N)	DLBCL (Rit, Cyc, Epi, V, P)	Asymptomatic	CT: no abnormal bowel dilatation or bowel wall thickening	NA	No antifungal agent given; stable
10 (C)	M/66 (J8N)	Mantle cell lymphoma (Cyc, Car, E)	Asymptomatic	Not done	NA	Posa 8 days after isolation of *Rhizopus sp.* in stool; stable
11 (C)	F/55 (J8N)	B-cell lymphoma (Cyc, Car, E)	Asymptomatic	Not done	NA	Posa 2 days after isolation of *Rhizopus sp.* in stool; stable
12 (M)	F/59 (K20N)	AML (Cyt, D)	Diarrhea	CT: mural thickening at cecum and terminal ileum	NA	Posa, AmB, Caspo 1 day after symptom onset; stable

[a] C, colonization case; I, invasive case; M, mucosal case; !, retrospectively identified.
[b] M, male; F, female. Ages are expressed in years.
[c] A, asparaginase; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATG, antithymocyte globulin; B, busulfan; Car, Carmustine; Clo, clofarabine; Cyc, cyclophosphamide; Cyt, cytarabine; D, daunorubicin; Dexa, dexamethasone; DLBCL, diffuse large B-cell lymphoma; E, etoposide; Epi, epirubicin; F, fludarabine; Ida, idarubicin; Met, methotrexate; MDS, myelodysplastic syndrome; Leu, leunase; P, prednisolone; Rit, rituximab; T, thioguanine; TBI, total body irradiation; V, vincristine.
[d] ALT, alanine aminotransferase (U/liter); ANC, absolute neutrophil count (10^9/liter); Cr, creatinine (μmol/liter); RLQ, right lower quadrant.
[e] CT, computer tomography; R, right.
[f] NA, not applicable.
[g] BMT, bone marrow transplant; AmB, amBisome; Caspo, caspofungin; Des, deferasirox; Posa, posaconazole.

TABLE 2. Characteristics of patients with or without intestinal zygomycosis

Characteristic	Value for group		P value[a]
	Case	Control	
Total	9	45	
Median age, yr (range)	50 (11–73)	46 (19–70)	0.552
Sex (male)	7	25	0.283
Underlying disease			0.512
Acute leukemia	5	23	
Lymphoma	3	13	
Myeloma	0	6	
Myelodysplastic syndrome	1	1	
Severe aplastic anemia	0	2	
Bone marrow transplantation	4	9	0.195
Median (range) hematological parameters on admission			
Absolute neutrophil count (10^9/liter)	2.3 (0.3–29.0)	2.1 (0.0–8.7)	0.710
Hemoglobin (g/dl)	11.1 (4.6–13.5)	10.7 (6.2–14.6)	0.642
Platelet count (10^9/liter)	145 (7–267)	157 (7–443)	0.410
Median (range) biochemical parameters on admission			
Urea (mmol/liter)	4.8 (2.9–11.5)	4.9 (2.0–13.5)	0.816
Creatinine (μmol/liter)	67 (50–106)	67 (43–140)	0.710
Random glucose (mmol/liter)	5.6 (4.2–11.6)	5.9 (4.4–8.9)	0.794
Albumin (g/liter)	41 (26–45)	40 (20–48)	0.584
Globulin (g/liter)	31 (20–41)	31 (23–63)	0.592
Total bilirubin (μmol/liter)	9.0 (3.0–13.0)	7.5 (2.0–42.0)	0.934
Alanine transaminase (U/liter)	32 (8–103)	26 (7–66)	0.508
Aspartate transaminase (U/liter)	39 (15–90)	25 (16–58)	0.068
Lactate dehydrogenase (U/liter)	228.5 (139.0–4,300.0)	195.0 (97.0–1199.0)	0.563
Epidemiological exposure			
Intake of allopurinol in hospital (yes/no)	9/0	9/36	<0.001
Intake of specific ready-to-eat food (yes/no)	7/2	9/36	<0.001
Use of wooden tongue depressor (yes/no)	0	0	NA
Use of wooden chopstick (yes/no)	0	0	NA

[a] NA, not applicable.

ples positive for *Mucorales* (one isolate [each] of *Rhizopus oryzae* and *Mucor* species, and three isolates of *R. microsporus*) from food specimens, and 16 samples positive for *Rhizopus* species from drug specimens. Further analysis demonstrated that all 16 samples of allopurinol tablets, 3 prepackaged ready-to-eat food items, and 1 pair of wooden chopsticks were positive for *R. microsporus.* None of the patients had ever used wooden chopsticks, and therefore, this factor was not included in further analysis. The mean viable fungal counts of allopurinol tablets obtained from ward and pharmacy were 4.22×10^3 CFU/g of tablet (range, 3.07×10^3 to 5.48×10^3) and 3.24×10^3 CFU/g of tablet (range, 2.68×10^3 to 3.72×10^3), respectively, whereas the mean viable counts in the food items were about 2×10^2 CFU/g.

The Hong Kong government and the manufacturer, which is a local pharmaceutical company, were notified of this important public health incident involving *Rhizopus*-contaminated allopurinol. All production lines of this pharmaceutical company were immediately suspended for further investigation. The raw materials and finished tablets of allopurinol were retrieved for microbiological culture by our laboratory and the government laboratory. Territory-wide recall and suspension of the use of this brand of allopurinol tablets were urgently implemented. Subsequent laboratory tests also showed that the cornstarch used in the excipients for allopurinol was found to contain 2 CFU/g of *R. microsporus*. During the site visit, it was found that the active and inactive ingredients of the drug granules were mixed and heated up to 50°C in an oven for 4 h and subsequently held at 20°C with a 3% water content for 5 to 14 days. This prolonged holding time was due to inevitable delays as a result of recent overbooking of the tableting machine. Such a long holding time provided an excellent opportunity for this thermotolerant fungus to multiply to a high level.

In view of this unusual occurrence of fatal cases due to intestinal zygomycosis in our hospital, the Hospital Authority of Hong Kong initiated a retrospective review and a 2-week prospective enhanced surveillance for all hospitalized hematology patients at all regional hospitals in Hong Kong. Ten other patients with intestinal infection by *R. microsporus* were identified in four regional hospitals. Two patients who succumbed in two other regional hospitals were confirmed to have invasive intestinal zygomycosis due to *R. microsporus* by postmortem histopathological and microbiological examination.

Laboratory identification of *Rhizopus* isolates. All patient-derived strains were identified as *R. microsporus* by their morphological appearance of fluffy, rapidly growing mold with aseptate, broad, readily twisted, ribbon-like hyphae, the presence of rhizoids directly under sporangiophores with unbranched sporangiophores, sporangium size of less than 100 μm, sporangiophore length of less than 1,000 μm (Fig. 2a to c), the absence of azygospores, and optimal growth at 42°C. *R. microsporus* var. *chinensis* and *R. microsporus* var. *rhizopodiformis* were differentiated by the spore appearance on electron microscopy (Fig. 2d to e). However, morphological transitions of spores between *R. microsporus* var. *chinensis* and *R. microsporus* var. *rhizopodiformis* were observed in the purified colo-

TABLE 3. Screening of food and drug samples for *Rhizopus microsporus* group

Item (n^a)	Description of sample (total no. and site of collection)[b]
Fresh fruits (2)	Apple (1 from kitchen)
	Orange (1 from kitchen)
Ready-to-eat food (179)	Additives (14 packs from kitchen),
	Biscuits (70 packs from kitchen and 12 packs from convenience store)*
	Cakes (5 from kitchen and 12 from convenience store)
	Cereals (3 packs from kitchen)
	Macaroni (1 pack from kitchen)
	Raisins (1 pack from kitchen)
	Sandwiches (3 from kitchen and 38 from convenience store)*
	Juice (2 bottles from kitchen and 1 bottle from convenience store)
	Milk (15 bottles from kitchen and 2 bottles from convenience store)
Drugs (150)	Acyclovir tablets (3 vials from wards, 14 vials from pharmacy)
	Allopurinol tablets (5 vials from wards, 11 vials from pharmacy)*
	Bactidol mouthwash (11 bottles from wards)
	Chinese herbal medicine (6 bottles from patients)
	Ciprofloxacin tablets (5 vials from pharmacy)
	Cotrimoxazole tablets (4 vials from wards, 6 vials from pharmacy)
	Dexamethasone tablets (6 vials from pharmacy)
	Esomeprazole (3 vials from wards, 21 vials from pharmacy)
	Famotidine tablets (1 vial from ward, 8 vials from pharmacy)
	Gastrocaine (1 vial from ward)
	Itraconazole tablets (1 vial from ward, 5 vials from pharmacy)
	Itraconazole syrup (1 vial from ward, 1 vial from pharmacy)
	Lactulose (1 vial from ward)
	Paracetamol (3 vials from wards, 11 vials from pharmacy)
	Phenytoin (2 vials from wards, 11 vials from pharmacy)
	Potassium syrup (2 vials from wards)
	Prednisolone (2 vials from wards, 5 vials from pharmacy)

[a] *n*, total no. of samples.
[b] *, growth of *Rhizopus* species.

nies under electron microscopy, which rendered the differentiation useless for typing purposes (Fig. 2f).

Antifungal susceptibility and treatment outcome. Susceptibilities of 12 patient-derived *R. microsporus* isolates to amphotericin B and posaconazole were determined by Etests. The MICs of amphotericin B and posaconazole ranged from 0.06 μg/ml to 1.5 μg/ml and 0.5 μg/ml to 2 μg/ml, respectively. The MICs of the control strain of *A. flavus* ATCC 204304 against amphotericin B and posaconazole were 0.5 μg/ml and 0.25 μg/ml, respectively.

Based on these MIC results and the previous reports of in vitro synergism between liposomal amphotericin B (AmBisome) and posaconazole (34) and between caspofungin and posaconazole (11), our patients were treated with these three antifungal agents, either in combination or with posaconazole alone, in addition to surgical intervention where possible, depending on the extent of involvement (Table 1). For the five patients with invasive intestinal disease, three (cases 1, 2, and 5) were identified prospectively and were given the combination therapy. Cases 1 and 5 succumbed despite surgery and use of antifungal agents due to the extensive involvement and failure of neutrophil recovery, whereas case 2 survived with an appendectomy, antifungal therapy, and recovery of neutrophils. The other two patients with invasive intestinal disease (cases 3 and 8) were identified retrospectively and were treated with fluconazole and voriconazole, respectively, as empirical antifungal therapy. The two patients (cases 4 and 12) with mucosal zygomycosis were treated with combination therapy (case 4 received anidulafungin instead of caspofungin), and both responded clinically with microbiological clearance of *Rhizopus microsporu*s in subsequent stool samples. One of them (case 4) succumbed 44 days after the onset of symptom due to severe veno-occlusive disease with liver failure and nonengraftment. All except one (case 9) of the five patients (cases 6, 7, 9, 10, and 11) with asymptomatic colonization were treated with posaconazole because of upcoming chemotherapy or conditioning for bone marrow transplant. All, including the patient who did not receive treatment, remained asymptomatic, with documented clearance of *R. microsporus* in subsequent stool cultures. After the use of this contaminated brand of allopurinol was stopped by switching to that produced by another manufacturer and after the strict enforcement of taking only boiled food items by this group of patients, there were no reports of any further cases in the subsequent 4 months of surveillance.

ITS sequencing. PCR of the ITS region of the fungal isolates produced amplicons of about 600 bp. Sequencing of the amplicons showed unambiguous sequence in 24 isolates. Phylogenetic analysis showed that there was >98% nucleotide identity between the sequence of any of the 24 isolates and those of *Rhizopus microsporus* var. *rhizopodiformis* (DQ641306), *Rhizopus azygosporus* (DQ119008), *Rhizopus homothallicus* (AF115728), and *Rhizopus oryzae* (AB097378). Among the 24 isolates, there were 8 sequence types (Fig. 3). The first one consisted of four patient isolates (P2, P3, P6, and P7), five drug isolates (D1-1, D1-2, D1-3, D2, and D3-2), two food isolates (F3 and F5), and one cornstarch isolate (R1); the second one consisted of two patient isolates (P8 and P9), one drug isolate (D4-2), and two food isolates (F4-1 and F4-2); the third one consisted of two food isolates (F2-1 and F2-2); and the other five consisted of one isolate each (P1, P4, P5, P10-1, and D5).

RAPD. PCR was performed with four random primers (OPC-01, TTCGAGCCAG; OPC-02, GTGAGGCGTC; OPC-11, AAAGCTGCGG; OPC-19, GTTGCCAGCC). Each set of reaction was performed in duplicate. Weak and indeterminate bands were excluded from the numerical analysis. For the *R. microsporus* isolates P9 and F1, we failed to produce consistent band patterns using the above primers and hence they were excluded from RAPD analysis. A total of 61 RAPD markers were established from the present study (unpublished data). We attempted to type the isolates by RAPD analysis, with limited evidence of clonal distribution.

DISCUSSION

We have reported the first major outbreak of intestinal infection by *R. microsporus* in patients with hematological ma-

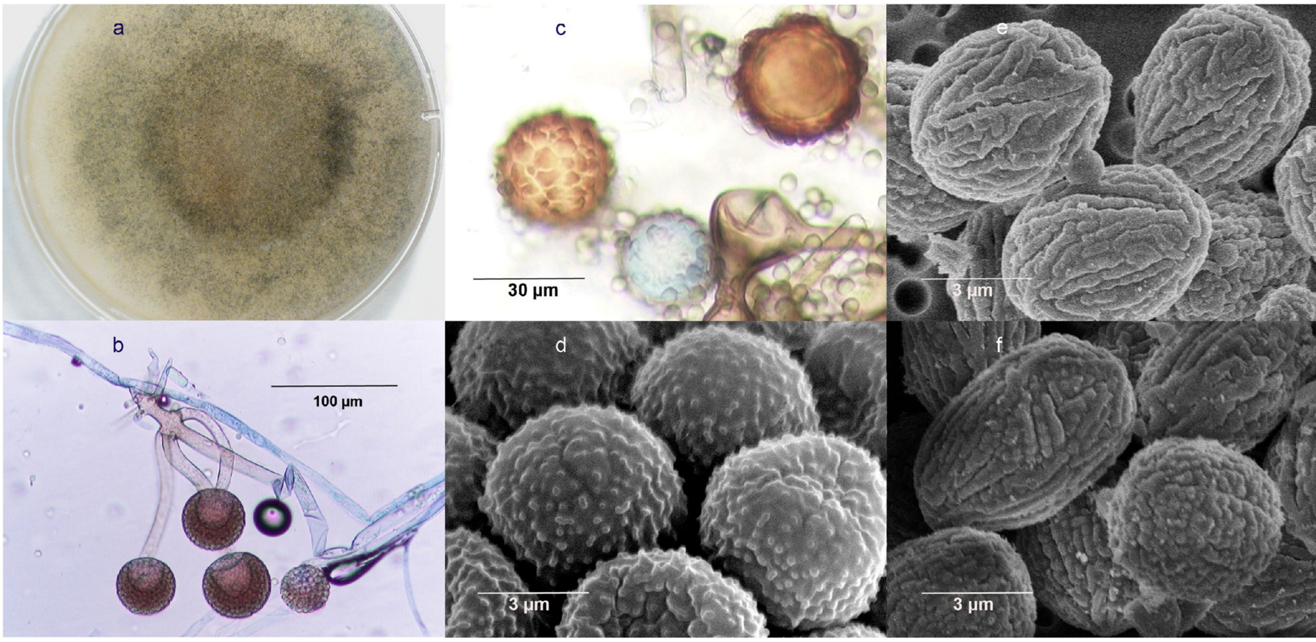

FIG. 2. (a) Clinical specimen was cultured for 48 h at 37°C; a fluffy colony with a pale gray color filled up the whole Sabouraud dextrose agar plate (80 mm). (b) With lactophenol cotton blue preparation, globose sporangia (up to 80 μm in diameter) were found with simple rhizoids directly under unbranched, short (up to 500 μm in length) sporangiophores. (c) In three-week-old culture, azygospores (up to 30 μm in diameter) were not found in all patient, food, and drug isolates except in the control strain. (d) For scanning electron microscopy examination of spore wall ornamentation, *R. microsporus* var. *rhizopodiformis* was observed as small, regularly spaced, and cylindrical spines formed on the wall of globose spores. *R. microsporus* var. *rhizopodiformis* was identified in patients' isolates (P3, P7, and P9) and in allopurinol (D2). (e) *R. microsporus* var. *chinensis*; rows of blunt warts were arranged mainly in line from pole to pole on the wall of ellipsoidal spores. *R. microsporus* var. *chinensis* was identified in patients' isolates (P1, P2, P4 to P6, P8, P10, and P11), food isolates (F3 and F4), and raw material (cornstarch) of allopurinol (R1). The presence of *R. microsporus* var. *chinensis* and that of *R. microsporus* var. *rhizopodiformis* were found simultaneously in isolates from patient (P12) and allopurinol (D1, D3, and D4). (f) Morphological transition of spores between *R. microsporus* var. *chinensis* and *R. microsporus* var. *rhizopodiformis* was observed in the purified colonies by electron microscopy.

lignancies and bone marrow transplantation. *Rhizopus* spp. have a worldwide distribution and are found in soils from cultivated grassland and forest locations. This organism has been isolated from a variety of food items, including corn, barley, sorghum, wheat, oats, rice, onions, groundnuts, sweet potatoes, pecans, and tomatoes (1, 36). However, reports on *R. microsporus*-related infections are relatively uncommon compared with reports on infections related to other *Rhizopus* species. It has been associated with moldy lumber, as evidenced by the presence of serological responses to *Rhizopus* species among sawmill workers (37, 38). It has also been found in the wooden material used for patient care. The use of contaminated wooden tongue depressors as splinters for intravenous catheter fixation in premature neonates (26) and wooden applicators for the preparation of drugs administered via the nasogastric tubes in critically ill patients (23) has been incriminated in nosocomial outbreaks of cutaneous and gastric zygomycosis, respectively. A pseudoepidemic has also been reported among patients with hematological conditions due to the use of contaminated wooden sticks during stool collection (57). To the best of our knowledge, an outbreak of *R. microsporus* due to contaminated oral medication and less likely food items, as shown in our case control and molecular analysis, has not been reported in the literature. Since the phylogenetic tree of ITS showed that the patient-derived isolates closely cluster with both drug and food isolates, it is not possible to blame either as the sole common source for this outbreak. However, the mean viable fungal counts in allopurinol tablets were much higher than the European Pharmacopoeia standard of less than 100 fungal counts per 1 g or 1 ml of nonsterile pharmaceuticals products (4, 10), whereas the mean viable fungal count in the positive food items were much lower. Upon further investigation of the source of drug contamination (31), the allopurinol tablet was found to contain cornstarch as part of its excipient ingredients, which contained 2 CFU/g of *R. microsporus*. During the time of dehydration of the granulations containing both active and inactive ingredients, the mixture was processed at 50°C in an oven for 4 h, with the subsequent holding at 20°C with 3% water content for 5 to 14 days. This long holding time provided an excellent opportunity for the fungus to multiply to a high level within this starch-containing mixture, which is nutritionally favorable for *R. microsporus*. Moreover, the thermotolerance of the fungus allows its survival at 50°C. Allopurinol and colchicine are commonly used in patients with hyperuricemia and gout. Their side effects are mainly related to hypersensitivity and drug interactions (16). Infective complications due to contamination have never been reported. After these cases were reported from our hospital, 10 similar cases were then found in other hospitals in Hong Kong.

At the moment, there is no standard typing method for zygomycetes, which poses severe limitations for the study of their molecular epidemiology (45). In a recent study reporting

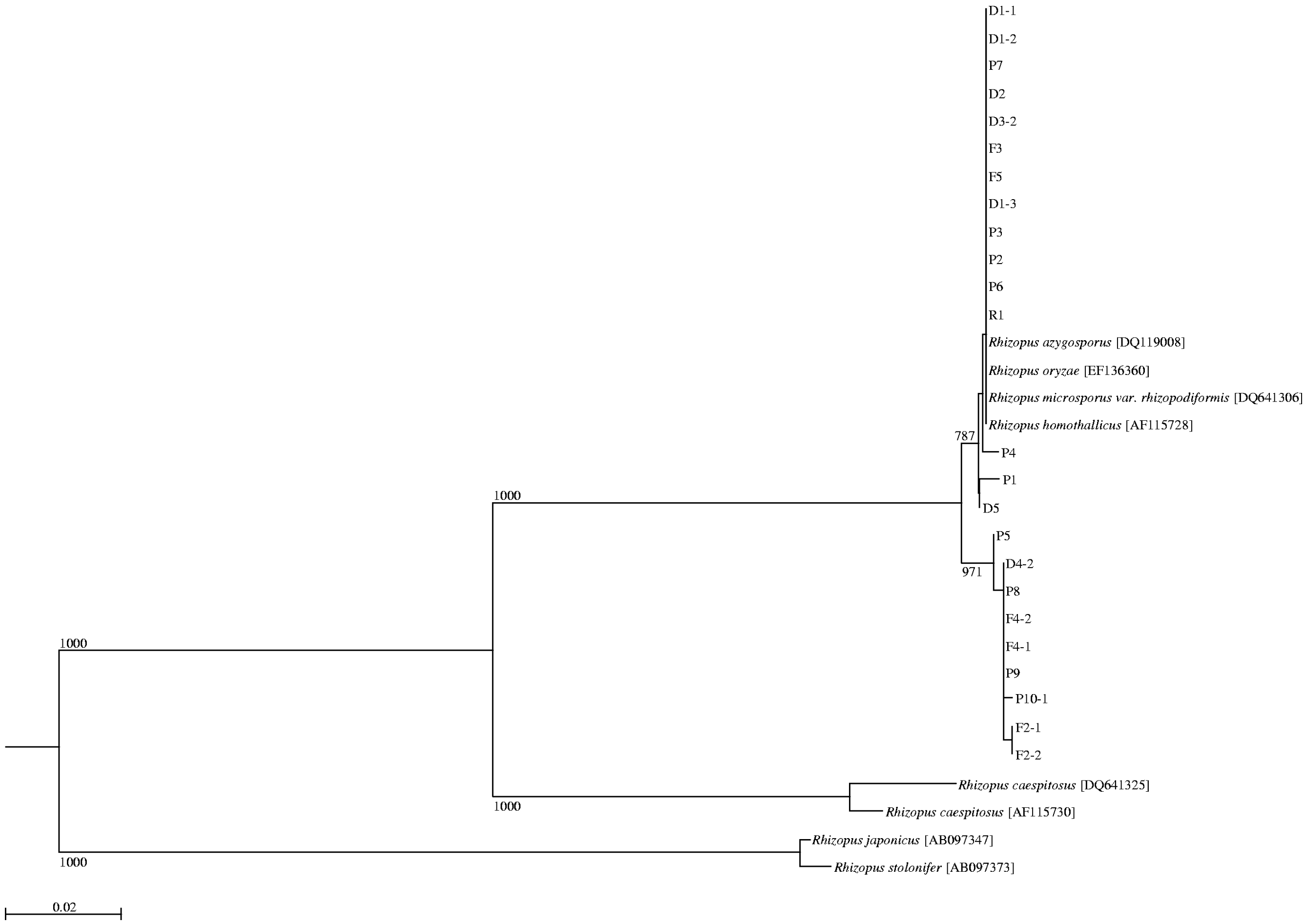

FIG. 3. Phylogenetic tree showing the relationship of 24 strains isolated from patients, foods, drugs and cornstarch, inferred from ITS (648 nucleotide positions) sequence data by the neighbor-joining method and rooted using sequence of *Pirella circinans* (AM778602). The scale bar indicates the estimated number of substitutions per 50 bases. Numbers at nodes indicated levels of bootstrap support calculated from 1,000 trees. All names and accession numbers are given as cited in the GenBank database.

27 cases of zygomycosis in a tertiary-care cancer center in the United States (21), amplification of repetitive genomic sequences adopted from *Aspergillus* typing was used (13). However, the strains of zygomycetes can be differentiated only at the genus level. Although RAPD analysis was applied in the case of *Rhizopus stolonifer* (55), the molecular typing of *R. microsporus* had never been reported in the literature. In this study, we actually tested more primers than reported, but some were excluded because of the lack of variability or reproducibility in pattern. This is consistent with the practice of other research using RAPD as a tool to differentiate *rhizopus* and *rhizomucor* species (55, 56). Our presently reported typing method by ITS sequencing or RAPD typing can differentiate our isolates into many types, but its usefulness still awaits further validation. In future outbreaks, more data can be accumulated to determine the cutoff criteria for clonality. At the moment, primer sets used in this study appear to be most discriminative and reproducible. At this stage, it is not possible to prioritize between ITS sequence typing and RAPD typing. PCR sequence analysis for one locus, such as ITS, is easily reproducible, comparable between laboratories, and illustrative of evolutionary relationships but less discriminative for clonality. RAPD involves PCR of multiple loci, which does not require sequencing but is less reproducible, and results are not easy to compare between laboratories. RAPD usually has greater discriminatory power, but it shows clustering rather than evolutionary relationships. For ITS sequencing, even one nucleotide difference can be considered unique because of fidelity of DNA polymerase in eukaryotes. As for RAPD, there are no interpretive standards for zygomycetes. Unlike the typing method for bacteria (19), the interpretative standards are based on large-scale comparison of many different strains with other typing methods; such a scale is not available for most molds at this stage.

Gastrointestinal manifestation has been rare in patients with zygomycosis. It has mostly occurred in premature neonates and is often associated with disseminated diseases (8, 18, 35, 41). Necrotizing enterocolitis was found in premature neonates (29, 43, 63) and less frequently in neutropenic adults (47, 48). All of our five cases of invasive intestinal zygomycosis had severe necrotizing ileocecitis leading to intestinal perforation with or without retroperitoneal necrosis, which was in contrast to the classical reports of the stomach being the most frequently involved site in adults with gastrointestinal zygomycosis (32).

Results in a recent study implied that the suppression of gastric acidity by proton pump inhibitors in critically ill patients might serve as a predisposing factor for gastric infection (23). Since most of our patients did not have concomitant pulmonary, rhinocerebral, or disseminated involvement, this suggested that the route of acquisition is oral ingestion rather than inhalation of fungal spores. Furthermore, the infecting dose was likely to be rather substantial, because in at least two of our cases (cases 1 and 2), the onset of invasive diseases occurred immediately after the first cycle of induction chemotherapy for acute leukemia. This phenomenon was also shown in a recent case report (40). Since the duration of the neutropenic phase was less than 2 weeks in our cases (cases 1 and 2), the risk of invasive fungal infection should not have been high unless there was a significant load of microbial exposure (14).

Zygomycosis has become an emerging infectious disease for immunocompromised patients. Prior to this outbreak, zygomycosis was uncommon in our locality (65). According to our laboratory information system, more than 140,000 fungal cultures were performed in 15 years, only 11 patients had culture-proven zygomycosis with respiratory or cutaneous involvement, and *Rhizopus* spp. had been encountered in only 2 patients before this outbreak. In a recent epidemiologic study involving 16 bone marrow transplant centers in North America, zygomycosis was found in 18 (7.2%) out of 250 episodes of invasive fungal infection among adult bone marrow transplant recipients between 2004 and 2007. *Rhizopus* species were found in 10 of these episodes (28). There was also a trend of increasing proportion of mold infection by zygomycetes (24). This phenomenon may be related to the introduction of voriconazole, a broad-spectrum antifungal agent, as a prophylactic regimen during bone marrow transplantation (25, 28, 44). In our bone marrow transplant center, voriconazole had not been used as routine antifungal prophylaxis. Thus, the emergence of zygomycosis in our locality at this stage is not related to selection by voriconazole. This phenomenon is therefore dissimilar to the emergence of *Candida krusei* or *Candida glabrata* after routine fluconazole prophylaxis in this group of patients (12).

The clinical spectrum of intestinal infection by *R. microsporus* ranges from asymptomatic colonization to mucosal involvement to invasive disease as illustrated in this outbreak. Our enhanced surveillance of stool culture picked up six additional patients (cases 6, 7, and 9 to 12) with asymptomatic colonization by *R. microsporus* and one other (case 4) with mucosal involvement as evidenced by segmental thickening of the proximal jejunal wall shown in a computerized tomography study. In contrast to the previous reports of invasive disease (2, 3, 20, 42), the organism could act as a colonizer or produce mucosal invasion in the gastrointestinal tract in the immunocompromised host, similar to that of *Candida* species. However, further investigation should be conducted to understand the natural evolution of clinical progression from colonization to invasion and whether there is any intracellular latency of *Mucorales* in the gastrointestinal tract in order to determine the optimal treatment and preventive strategy for this new clinical entity.

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